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(REV 11-98)

U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

ATTORNEY'S DOCKET NUMBER

TRANSMITTAL LETTER TO THE UNITED STATES

101195-45

DESIGNATED/ELECTED OFFICE (DO/EO/US)

U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR

CONCERNING A FILING UNDER 35 U.S.C. 371

TBA 09/786926

INTERNATIONAL APPLICATION NO.
PCT/DE99/02871INTERNATIONAL FILING DATE
10 Sept. 1999 (10.09.99)PRIORITY DATE CLAIMED
11 Sept. 98 (11.09.98)

TITLE OF INVENTION

Human and Murine G-protein-coupled Edg6 Receptor (Endothelial Differentiation Gene) and Use of Same

APPLICANT(S) FOR DO/EO/US

Markus Graler; Gunter Hardt; and Martin Lipp

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This is an express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. ☐ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. ☒ A copy of the International Application as filed (35 U.S.C. 371 (c) (2))
 - a. ☐ is transmitted herewith (required only if not transmitted by the International Bureau).
 - b. ☒ has been transmitted by the International Bureau.
 - c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. ☐ A copy of the International Search Report (PCT/ISA/210).
8. ☐ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3))
 - a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
 - b. ☐ have been transmitted by the International Bureau.
 - c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
 - d. ☐ have not been made and will not be made.
9. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
10. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371 (c)(4)).
11. ☐ A copy of the International Preliminary Examination Report (PCT/IPEA/409).
12. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).

Items 13 to 20 below concern document(s) or information included:

13. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
14. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
15. ☒ A **FIRST** preliminary amendment.
16. ☐ A **SECOND** or **SUBSEQUENT** preliminary amendment.
17. ☐ A substitute specification.
18. ☐ A change of power of attorney and/or address letter.
19. ☒ Certificate of Mailing by Express Mail
20. ☐ Other items or information:

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|--|--|--|--|--|--|
| U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR 1.53) 09/786926 | | INTERNATIONAL APPLICATION NO. PCT/DE99/02871 | | ATTORNEY'S DOCKET NUMBER 101195-45 | |
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21. The following fees are submitted:

| BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)) : | | | | CALCULATIONS PTO USE ONLY | |
|---|---|--------------|--------------------------|-------------------------------------|-------------------|
| <input type="checkbox"/> | Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO | \$1,000.00 | | | |
| <input checked="" type="checkbox"/> | International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO | \$860.00 | | | |
| <input type="checkbox"/> | International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO | \$710.00 | | | |
| <input type="checkbox"/> | International preliminary examination fee paid to USPTO (37 CFR 1.482) but all claims did not satisfy provisions of PCT Article 33(1)-(4) | \$690.00 | | | |
| <input type="checkbox"/> | International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(1)-(4) | \$100.00 | | | |
| ENTER APPROPRIATE BASIC FEE AMOUNT = | | | | | |
| Surcharge of \$130.00 for furnishing the oath or declaration later than <input type="checkbox"/> 20 <input checked="" type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492 (e)). | | | | | |
| CLAIMS | NUMBER FILED | NUMBER EXTRA | RATE | | |
| Total claims | 20 - 20 = | 0 | x \$18.00 | | \$0.00 |
| Independent claims | 8 - 3 = | 5 | x \$80.00 | | \$400.00 |
| Multiple Dependent Claims (check if applicable). | | | <input type="checkbox"/> | | \$0.00 |
| TOTAL OF ABOVE CALCULATIONS = | | | | | \$1,390.00 |
| Reduction of 1/2 for filing by small entity, if applicable. Verified Small Entity Statement must also be filed (Note 37 CFR 1.9, 1.27, 1.28) (check if applicable). | | | | <input checked="" type="checkbox"/> | \$695.00 |
| SUBTOTAL = | | | | | \$695.00 |
| Processing fee of \$130.00 for furnishing the English translation later than <input type="checkbox"/> 20 <input checked="" type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492 (f)). | | | | + | \$130.00 |
| TOTAL NATIONAL FEE = | | | | | \$825.00 |
| Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31) (check if applicable). | | | | <input type="checkbox"/> | \$0.00 |
| TOTAL FEES ENCLOSED = | | | | | \$825.00 |
| | | | | Amount to be: refunded | \$ |
| | | | | charged | \$ |

- ☐ A check in the amount of _____ to cover the above fees is enclosed.
- ☒ Please charge my Deposit Account No. **14-1263** in the amount of **\$825.00** to cover the above fees.
A duplicate copy of this sheet is enclosed.
- ☒ The Commissioner is hereby authorized to charge any fees which may be required, or credit any overpayment to Deposit Account No. **14-1263** A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

the correspondence address associated with Customer No. 27387



27387

PATENT TRADEMARK OFFICE

SIGNATURE

Bruce S. Londa

NAME

33-531

REGISTRATION NUMBER

March 9, 2001

DATE

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JG08 Rec'd PCT/PTO 09 MAR 2001

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Atty's Docket No. 101195-45

APPLICANT : Markus Graler et al.
FILED : Concurrently Herewith
FOR : Human and Murine G-protein-coupled Edg6
Receptor (Endothelial Differentiation Gene) and
Use of Same

PRELIMINARY AMENDMENT

Hon. Assistant Commissioner of Patents
Washington, D.C. 20231

Sir:

Prior to examination, please amend the application as
follows:

IN THE CLAIMS

Please make claim 7 dependent solely on claim 3;

Please make claim 15 dependent solely on claim 15;

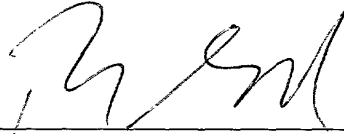
Please make claim 20 dependent solely on claim 18.

09/786926-050404

REMARKS

The above amendments were made to remove the multiple dependencies.

Respectfully Submitted,



Bruce S. Londa
Attorney for Applicant
Norris, McLaughlin & Marcus P.A.
220 East 42nd Street, 30th Floor
New York, N.Y. 10017
Telephone: (212) 808-0700
Telecopier: (212) 808-0844

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G-protein coupled receptor EDG6 and its use

Description

The invention in question is concerned with the G-protein coupled receptor EDG6 and its fragments, variants and mutations as well as its use. Fields of application of the invention are molecular biology, pharmacy and medicine.

The super-family of the G-protein coupled receptors (GPR's) contains several hundred proteins. Their principal task in the organism entails forwarding information from the extra-cellular environment to the inner of the cell through interaction with heterotrimer guanine nucleotide binding proteins, commonly known as G-proteins (Dohlman et al., 1987). In this way, they have an influence on the regulatory processes inside cells (Böhm et al., 1997).

The large number of GPR's is also reflected in a great variety of extra-cellular ligands, the so-called 'first messengers'. GPR's for hormones and neurotransmitters are known, for paracrine substances and inflammatory mediators, for certain proteases, for a large number of taste and smell molecules as well as for photons and calcium ions (Watson and Arkinstall, 1994).

The EDG (endothelial differentiation gene) receptor family belongs to the group of the G-protein coupled receptors (GPR's). The first member of this family, EDG1, was cloned in 1990 in the course of an examination of the non-proliferative aspects of angiogenesis in the organisation and differentiation of endothelium cells in capillaries.

A further member of the EDG receptor family, EDG2, is found above all in cortical neurogenic regions.

The cDNA of a third member of the EDG receptor family was isolated from human placenta, kidney and liver and from human heart and localised to chromosome 9q22.1-2. Human EDG4 cDNA transcripts have recently been proven from testes, prostate, pancreas and peripheral blood leukocytes and, to a lesser extent, in the thymus and spleen. Two further transcripts have been isolated from smooth muscle cells of the rat aorta and from murine and bovine taste buds. Lysophosphate idyl acid (LPA; 1-Acyl-2-hydroxy-*sn*-glycero-3-phosphate) has been identified as a possible ligand for the human and the murine EDG2 receptor and also for the human EDG4 receptor. Further, lysosphingolipids have been found as functional ligands for the human receptors EDG1 and EDG3 and for the H218 receptor of rats.

The invention was based on the task of isolating and identifying a further member of the EDG receptor family and making it useful for a medicinal application.

It was seen that *in vitro* differentiated dendritic cells express a further EDG receptor, which is termed as EDG6.

The human EDG6 receptor entails 384 amino-acids of Sequence 1 with seven transmembrane domains. The receptor possesses a possible N-terminal glycosylation point, three possible palmitoylation points positioned 12 to 15 amino-acids C-terminally from the seventh transmembrane domain as well as four possible C-terminal proteinkinase C-phosphorylation points. A model of the receptor is shown in Figure 1.

The EDG6 receptor is the first member of the EDG family to be isolated from *in vitro* differentiated human dendritic cells. In the Northern Blot analysis, it shows a signal at about 1.7 kb. Human *edg6* mRNA is expressed in the Burkitt lymphoma cell lines JBL2, BL64 and DG75, in the pro-myelocytic cell line U937 and in the T cell line CEM. High tissue-specific expression rates of human *edg6* have been found in human adult and foetal spleen and

in adult peripheral leukocytes and the lung. Lower expression rates of human *edg6* have been detected in adult thymus, lymph nodes, bone marrow and appendix as well as in foetal liver, thymus and lung.

The scope of the invention also includes the cDNA sequence of the EDG6 receptor with Sequence 2.

In the search for homologous sequences for sequence 2 in the nuclein acid database with the help of the BLASTN programme from the Husar package of the German Cancer Research Centre (DKFZ) in Heidelberg, a murine clone with high homologies to the newly identified EDG6 receptor was identified. The murine cDNA clone from the EST (expressed-sequence tag) nuclein acid database comes from lymph nodes and is 88 % homologous to the human EDG6 receptor for 106 amino-acids on an amino-acid basis after a correction of the database entry, which leads to an alteration of the reading frame. This means that this cDNA clone is a fragment of the murine homologue to the human EDG6 receptor.

Starting with this partial clone, the entire murine cDNA sequence (sequence 3) was determined with the help of a specific polymerase chain reaction.

The protein derived from this cDNA sequence has Sequence 4.

Further, specific anti-EDG-6 antibodies are also being claimed. They are produced by rats being immunised with a GST fusion protein containing a fragment of the EDG6 receptor. With the help of the rat's spleen cells, hybridomas are produced, which are examined for the production of specific antibodies by means of an ELISA analysis.

Further possibilities of obtaining antibodies are immunisation with whole cells which express the receptor EDG6 after inclusion

of the cDNA or also naturally, and also with a C-terminal 6 x histidin coupled N-terminal EDG6 fragment.

In the further extension of the invention, EDG6-deficient mice are produced, i.e. mice containing the function-free mutants ('zero mutants') of EDG6. These knock out mice act as an animal model for diseases possibly connected with the EDG6 receptor. The characterisation of the phenotype of these mice can make a decisive contribution to determining the function. For this, a function-free *edg6* gene with corresponding selection markers is integrated into the genome of murine embryonal stem cells (ES cells) with the help of homologous recombination. The selected ES cells are then inserted into in murine embryos of early cell development (morula, blastocysts) as multi-potent cells.

With these mouse strains, further statements can be made about the function of the EDG6 receptor, e.g. diseases directly or indirectly caused by the EDG6 receptor or reinforced in their extent can be established. In particular, this includes immune weaknesses, for example against infections, and diseases based on acute and chronic inflammations. Further, it also includes auto-immune diseases, allergies and malign diseases such as tumours, leukaemia and lymphomas. Due to the high homology of the murine receptor with the human receptor and also due to the highly preserved tissue-specific expression pattern, the results of the mouse model can be transferred to humans to a great extent. The human receptor EDG6 according to the invention can be directly or indirectly involved in the occurrence of immune weaknesses, acute and chronic inflammations, auto-immune diseases, allergies and malign diseases or strengthen their severity or extent.

Furthermore, the invention is also concerned with the use of the EDG6 nuclein acids and EDG6 polypeptides in original, modified or synthetic form as an initial basis for the development of pharmaceutically relevant substances. The EDG6 nuclein acids are used for the build-up of genes and vectors and the EDG6 polypeptides for the build-up of ELISA methods, measuring and

testing procedures. The pharmaceutically relevant substances either bind themselves or the receptor-coding nuclein acid to the receptor polypeptide or they influence the binding of the physiological ligands or the binding and activity of intracellular subsequent signal molecules, thus leading to an activation or inhibition of the receptor functions. These substances with an agonistic or antagonistic effect on the function of the EDG6 receptor can be organic molecules, inorganic molecules and peptides or combinations of these substance classes.

The invention enables a medicinal use for the following diagnostic or therapeutic measures.

1. In diseases caused by expression deviating from the norm or by receptor mutation, diagnostics can be done, e.g. with test kits on the basis on monoclonal antibodies or nuclein acid proof processes.

2. In diseases connected with the receptor function, the functions of the receptor can be influenced in an agonistic or antagonistic way.

3. The EDG6 receptor in an original or modified form as well as specific antibodies or binding partners can be used for therapeutic purposes, for example for gene-therapeutic processes (e.g. on a cellular, liposomal or viral basis) if a malfunction of the receptor or faulty expression of the EDG6 receptor or its ligands exists.

The invention is to be explained in more detail below by examples of embodiments.

Results

For the determination of new chemokine receptors and related receptors involved in the regulatory sequences of the immune

system, the polymerase chain reaction (PCR) with degenerated primers was used in order to identify G-protein coupled receptors (GPCRs) from *in vitro* differentiated human dendritic cells. Human peripheral mononuclear blood cells were differentiated to dendritic cells by treatment with the cytokines GM-CSF and IL-4. The PCR with degenerated primers from areas of the second and seventh transmembrane domain (TM) of some chemokine receptors led to the identification of a 648 bp fragment, which is part of a new member of the GPCR super-family. The possible full-length cDNA entailing 1560 bp was gradually cloned by means of 5' and 3' RACE-PCR. The cDNA contains an open reading frame of 1155 bp, a 5' non-translated region 22 bp in size as well as a 3' non-translated region 383 bp in size. However, the cDNA at the 3' end is possibly not complete, as it does not have a typical polyadenylation signal. Sequence 1 shows the resulting amino-acid sequence. Sequence comparisons indicate that the newly identified receptor belongs to the EDG family of the GPCRs. This is why it was called EDG6. The comparison of the amino-acid sequence of EDG6 with the other EDG receptor molecules from the first to the seventh transmembrane domain shows that EDG6 has 46% identity with EDG3, 44% with EDG1, 39% with EDG4 and 37% with EDG2. The nearest related GPCR is hCB1R, a member of the cannabinoid receptor family, with 31% identity. Thanks to computer-supported analyses, the possible localisation of the seven transmembrane domains, a possible N-glycosylation point in the N-terminal extra-cellular region and a number of post-translational modification points in the C-terminal cytoplasmatic domain were determined. Further, the correct orientation of the molecule in the cell membrane was examined in more detail with the N terminus on the extra-cellular side. For this, the protein-coding cDNA sequence was cloned into a eucaryotic expression vector, maintaining the reading frame, an *myc* epitope expressed to the C-terminal of the cloned sequence. After transfection of this vector into the human embryonal renal cell line HEK 293, the fusion molecule was merely detected in cells made permeable by an anti-myc specified monoclonal antibody by means of throughflow

cytometry. Further, the last 50 bp of the human *edg6* (*hedg6*) cDNA are identical with the base pairs 13 to 62 of a short sequence containing the repetitive dinucleotide polymorphism D19S120. This polymorphism has been localised to chromosome 19p13.3. With a PCR with gene-specific primers from *hedg6* cDNA and the D19S120 amplicon, a human genomic DNA fragment containing the 3' end of *hedg6* and the D19S120 polymorphism was amplified. Thus it is shown that *hedg6* is localised on chromosome 19p13.3 next to the D19S120 marker.

Further, the murine homologue of the *edg6* (*medg6*) cDNA was isolated with the help of the RACE-PCR. For this, overall RNA of cell line 18 from murine foetal skin was used. Gene-specific primers were produced, coming from the murine EST sequence of the cDNS clone va16c04.r1 (Gene Bank entry no. AA254425) and having high identity with the 3' end of the coding region of the *hedg6* cDNA. The primers were selected in such a way that the open reading frame of *medg6* could be amplified. For this reason, the *medg6* cDNA at the 3' end is incomplete. It contains an open reading frame of 1161 bp. The first 99 bp of the 5' non-translated region entailing 499 bp contain a murine repetitive element B1. The open reading frame of the *medg6* cDNA is 80% homologous with the corresponding human sequence. On a protein level, both sequences have an identity of 82% and a similarity of 91%. The possible post-translational modification points are preserved both in the human and also in the murine *edg6* sequence.

Next, the expression pattern of *edg6* was examined, for which DNA fragments were produced representing regions with low preservation in the murine and in the human cDNA sequence. These fragments were then inserted in Northern blots as radioactively marked sensors. In human cell lines, a *hedg6* specific signal was found at about 1.7 kb. *hedg6* mRNA is expressed in the Burkitt lymphoma cell lines JBL2, BL64 and DG75, in the pro-myelocytic cell line U937 and in the T-cell line CEM, whereas it is not detected in the oesophagus cancer cell line HEp2 and the HEp2

sub-clone cl32 and in the cervical carcinoma cell line HeLa. Generally, *hedg6* is weakly expressed in all the tested positive cell lines and can only be detected by extended exposure times of the blots. Due to the high specificity of the hybridisation samples, it is possible to determine the tissue-specific expression of *hedg6* with mRNA samples from 50 different human tissues by means of a dot blot. High expression rates of *hedg6* were found in human adult and foetal spleen and also in adult peripheral leukocytes and the lung. Lower expression rates of *hedg6* were detected in the adult thymus, lymph nodes, bone marrow and appendix and also in foetal liver, thymus and lung.

The tissue-specific expression of *medg6* mRNA in the range of the organs examined matches the human expression sample very well. Hybridisation signals were found in murine lung, spleen, thymus and lymph nodes, whereas there were none in non-lymphatic tissue. The murine *edg6* mRNA is about 2.1 kb in size and thus 0.4 kb larger than the human *edg6* mRNA.

Material and methods

Isolation of peripheral mononuclear blood cells

Peripheral mononuclear blood cells (PBMC) were obtained from fresh primary blood cells (buffy coats) by means of density centrifuging. To start with, 10 ml of the fresh primary blood cells were mixed with 20 ml of PBS each in 50 ml Falcon tubes. The PBS had been mixed with 5 U/ml of heparin. This mixture was added with 10 ml of Ficoll separation solution from the firm of Biochrom and centrifuged for 20 min at 200 x g. Then, the upper 20 to 25 ml of the mixture were removed. The rest of the mixture, which was still contaminated with thrombocytes, was then centrifuged once more for 20 min at 460 x g. The inter-phase of all the Falcon tubes formed was collected and washed three times

for 15 min at 300 x g with ice-cold PBS, mixed with 1 mM EDTA, in order to avoid a contamination with thrombocytes as far as possible.

5 x 10⁷ peripheral mononuclear blood cells were put into 3 sterile Petri dishes with a diameter of 10 cm together with 15 ml of RPMI medium and cultivated for 2 h in a CO₂ incubator at 37°C. Then, the base of the Petri dish was washed carefully from all sides a number of times with the RPMI medium by means of a glass pipette, with a majority of the non-adhering cells separating from the base. The non-adhering cells were thrown away together with the medium. After this, 15 ml of fresh RPMI medium heated to 37°C were added to each Petri dish, which now contained 800 U/ml of GM-CSF and 1000 U/ml of IL-4. From now on, the medium was refreshed a number of times every other day. In this, 7.5 ml of the medium were removed from each Petri dish and replaced by new RPMI medium, now containing 1600 U/ml of GM-CSF and 1000 U/ml of IL-4. On the 7th day of the cell culture, the cells were harvested.

The Burkitt lymphoma cell lines BL64 and DG75 as well as the lymphoblastoid T cell line CEM and the pro-myelocytic cell line U937 were cultivated in RPMI1640 medium with 10% foetal calves' serum, the oesophagus cancer cell line HEP2 and the HEP2 sub-clone cl32 as well as the human embryonal renal cell line HEK293 were cultivated in DMEM medium with 10% foetal calves' serum.

RNA isolation

Overall RNA was prepared with the TRIzol reagent of the firm of Gibco BRL according to the supplied protocol. The preparation of mRNA was done with the "Micro mRNA Purification Kit" of the firm of Pharmacia Biotech on the basis of the enclosed documents.

Northern blot

The transfer of the RNA was done according to the capillary blot method, which makes a purposeful transfer of RNA fragments by ion

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migration possible. In it, a pane of glass about as wide as the gel to be blotted was placed across a dish filled with 20x SSC buffer. Two filter papers of the length of the gel to be blotted and wide enough to protrude into the dish filled with buffers with both overlapping edges when lying across the pane of glass, were saturated with 20x SSC buffer and laid on top of one another on the glass pane in the way described. Then, the RNA gel on the top was placed to the bottom in a perfect match and free of bubbles. In the same way, the nitrocellulose membrane which has the size of the gel and had been placed in water and then in 20x SSC buffer beforehand, was placed on the gel. As the gel still contained a considerable amount of formaldehyde, the blot was set up under a subtraction. A water-impermeable plastic mask which enclosed the edges of the blot water-tight around the membrane was placed on the nitrocellulose membrane. Two further filter papers of the size of the nitrocellulose membrane were saturated in 20x SSC buffer and also positioned with a perfect match and free of bubbles. A pile of dry paper towels formed the upper edge of the set-up. Weighed down with a weight of about 0.5 kg, blotting was done for about 2 days. The Rna was fixed at 80°C for 2 hours.

For the hybridisation, a 32P marked cloned human or murine edg6 cDNA fragment was used. The marking reaction was done with the "Random Primed Labelling Kit" of the firm of Gibco BRL according to their instructions. The human RNA master blot of the firm of Clontech was hybridised and washed according to the documents supplied.

Polymerase Chain Reaction

One μg of the mRNA isolated from in vitro differentiated human dendritic cells was reversely transcribed with the reverse transcriptase "Superscript" of the firm of Gibco BRL in the presence of one pmol of a 25 to 30-mer oligo(dT)-primer. The PCR amplification by means of Thermoprime Plus DNA Polymerase of the firm of Advanced Biotechnologies was done with 100 pmol of the following primers: R1 (5'-C-CGG-ATC-CGC-VTD-VTS-GGM-AAY-KBV-YTS-

GT-3'), R3 (5'-CG-GGA-TCC-GAA-RGY-RTA-SAD-SAD-RGG-RTT-3'). Cycle: 94°C, 60 sec.; 48-63°C, 30 sec.; 72°C, 90 sec.; 35 cycles. For the amplification of the 3' and 5' ends of the human *edg6* cDNA, a RACE-PCR was carried out with the following primers: 5'hGSPRT (5'-TTG-GAG-CCA-AAG-ACG-TCG-GCC-3'), 5'-hGSP1 (5'-AGG-CAG-AAG-AGG-ATG-TAG-CGC-3'), 5'-hGSP2 (5'-GCG-CTC-CCC-TGC-AGT-GAA-GAG-3'), 3'-hGSP1 (5'-AGT-GAC-CTG-CTC-ACG-GGC-GCG-3'), 3'-hGSP2 (5'-CTC-TTC-ACT-GCA-GGG-GAG-CGC-3'). The reactions were carried out according to the protocol of M.A. Frohman (Frohman, 1995). The amplification of the 5' end of the murine *edg6* cDNA was likewise done with the help of the RACE-PCR with the following primers: 5'-mGSPRT (5'-CTC-ACC-TCG-TCT-GGG-AGG-GCC-TGC-3'), 5'-mGSP1 (5'-TGG-GCA-ACT-GGC-TGG-TCC-AAG-CTC-3'), 5'-mGSP2 (5'-GCC-TCG-GGC-CCA-GAT-CCT-CCA-GGG-GTG-CTG-CGG-ACG-CTG-GAA-ATG-CTG-G-3').

Before, as described above, a reverse transcription was done with 10 µg of overall RNA of the murine cell line 18. The 5'-mGSP2 primer contains a part of the myc-epitope sequence for further experiments. The primers were selected on the basis of the murine EST sequence of the cDNA clone *val6c04.r1* (Gene Bank entry no. AA254425), which has a high homology with the 3' end of the coding human *edg6* cDNA. The reactions were also carried out according to the protocol of M.A. Frohman (Frohman, 1995) with an additional cleaning step by means of "MicroSpin S-400 HR" columns of the firm of Pharmacia Biotech on the basis of the supplied protocol following the 5' polyadenylation reaction. Further, 10 µl of undiluted presentation DNA were used for both amplifications of the RACE-PCR. The murine *edg6* cDNA fragment, which was used in the Northern blot as a radioactively marked sample, was amplified as described above through the reverse transcriptase polymerase chain reaction from an overall RNA preparation of the murine cell line 18 with 25 pmol each of the 3' primer (5'-CCA-CGT-CCT-CCT-GCC-CGC-CGC-3') and 25 pmol of the 5'-mGSP2 primer (see above). Cycle: 94°C, 60 sec.; 50°C, 60 sec.; 72°C, 90 sec.; 35 cycles. The amplification of the genomic 3' sequence of the human *edg6* was done by means of PCR from 400 ng HEP2 genomic DNA with 25 pmol of the 3'-hGSP2 primer (see above)

and 25 pmol of the CA primer (5'-CCA-CTT-CCC-GCA-ACG-CCC-AGA-3'). Cycle: initial denaturing, 95°C, 5 min.; 95°C, 30 sec.; 60°C, 30 sec.; 72°C, 90 sec.; 30 cycles.

Cloning and sequencing

The cDNA fragments of the PCR reactions with the degenerated primers were cloned into the pZErO-2 vector of the firm of Invitrogen to Bam HI Verdau. The human edg6 RACE-PCR products were cloned into the same vector to HIND III/Pst I Verdau. They were ligated to a full-length clone at the Pst I interface. The murine edg6 5'-RACE-PCR product was cloned into the pZErO-2 vector to HIND III/Eco RV restriction. For this, the RACE-PCR product was HIND III-digested after a T4-polymerase reaction. The human cDNA fragment for the radioactive marking was isolated to Pst I/Aat II-restriction of the full-length clone (bp 438-842). The amplified murine cDNA fragment (bp 328-637) was cloned into the Apa I cut pZErO-2 vector. This fragment was used as a sensor in Northern blot after radioactive marking. All the fragments were sequenced with the "Thermo Sequenase fluorescent labelled primer cycle sequencing kit with 7-deaza-dGTP" of the firm of Amersham International and analysed with the help of the Li-Cor sequencer of the firm of MWG Biotech according to the supplied protocols.

Construction, expression and FACS analysis of the myc-epitope marked human EDG6 receptor

The construction of the C-terminal myc-epitope marked human EDG6 receptor and its expression in HEK293 cells and its analysis by means of throughflow cytometry was done as described (Emrich et al., 1993).

Computer analyses

Sequence comparisons, database research and statistical calculations were done with the help of HUSAR package V4.0 at the German Cancer Research Centre in Heidelberg and with the ClustalX V1.62b PC programme.

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Legend for the figures:

Figure 1: Schematic portrayal of a GPRs in the cell membrane (to Emrich, 1995). The arrangement of the (α -helical transmembrane domain (I-VII) is portrayed by cylinders. Possible glycosylation (••) and phosphorylation points (P) are marked, as is a possible palmitoylation point (\diamond).

Figure 2A: Northern blot with overall RNA of the human Burkitt lymphoma cell lines BL64 and DG75, of the pro-myelocytic cell line U937 and the lymphoblastoid T cell line CEM as well as with mRNA of the oesophagus cancer cell lines HEp2 and cl32, hybridised with a radioactively marked sensor of the human edg6 cDNA. Ethidium bromide dyed rRNA is shown as a control.

Figure 2B: Human RNA master blot (Clontech), hybridised with a radioactively marked sensor of the human edg6 cDNA. A1: testes; A2: ovaries; A3: pancreas; A4: hypophysis; A5: adrenal gland; A6: thyroid gland; A7: salivary gland; A8: mammary gland; B1: kidney; B2: liver; B3: small intestine; B4: spleen; B5: thymus; B6: peripheral leukocytes; B7: lymph nodes; B8: bone marrow; C1: appendix; C2: lung; C3: oesophagus; C4: placenta; D1: foetal brain; D2: foetal heart; D3: foetal kidney; D4: foetal liver; D5: foetal spleen; D6: foetal thymus; D7: foetal lung. No edg6-specific hybridisation signals were received from the mRNA of the following human tissues (not illustrated): entire brain, cerebellum, cerebral cortex, frontal lobes, hippocampus, pituitary gland, occipital lobes, putamen, Substantia Nigra, temporal lobes, thalamus, spinal cord, heart, aorta, skeletal muscle, large intestine, urinary bladder, uterus, prostate, stomach.

Figure 2C: Diagram of the relative intensity of the dot blot signals from selected organs.

Figure 2D: Northern blot with overall-RNA of murine organs, hybridised with a radioactively marked sensor of the murine edg6 cDNA. Ly: lymph nodes; sp: spleen; th: thymus; lu: lung; si: small intestine; li: large intestine; st: stomach. No edg6-specific hybridisation signal was received from the following overall-RNA preparations of murine tissue (not illustrated): heart, liver, kidney, skeletal muscle, pancreas, cerebellum, cerebrum.

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Patent claims

1. Human G-protein coupled receptor EDG6 with the sequence 1 as well as its fragments, variants and mutations.
2. Murine G-protein coupled receptor EDG6 with the sequence 4 as well as its fragments, variants and mutations.
3. DNA sequence coding the human G-protein coupled receptor EDG6 as well as its fragments, variants and mutations.
4. DNA according to claim 3, wherein there exists sequence 2.
5. DNA sequence coding the murine G-protein coupled receptor EDG6 as well as its fragments, variants and mutations.
6. DNA according to claim 5, wherein there exists sequence 3.
7. Vectors containing a DNA sequence, if applicable coupled to a suitable promoter in accordance with claims 3-6.
8. Host cells containing vectors according to claim 7.
9. Antibodies against human or murine EDG6 G-protein coupled receptors.
10. Monoclonal antibodies according to claim 9.

11. Test kit for detection of the EDG6 receptor on the basis of monoclonal antibodies according to claim 10.

12. Test kit for detection of the EDG6 receptor on the basis of nuclein acid diagnostics.

13. Use of the EDG6 receptor as well as its fragments, variants and mutations and, if applicable, its binding partners for therapeutic methods and treatments.

14. Use according to claim 13, wherein the therapeutic methods influence the function of blood and body cells, for example leading to the inhibition of chronic and acute inflammations.

15. Use of the EDG6 receptor as well as its fragments, variants and mutations and, if applicable, its binding partners according to claims 13 - 14, wherein there exists the use for gene-therapeutic methods and treatments.

16. Use of the monoclonal antibodies according to claim 10, if applicable coupled to other molecules and substances, for example therapeutic agents, toxins or antibodies, for therapeutic methods and treatments.

17. Use according to claim 16, wherein the therapeutic measures influence the function of the EDG6 receptor, for example in immune and inflammatory reactions.

18. EDG6-deficient mouse strains containing function-free mutants ('zero mutants') of the EDG6.

19. Mouse strains according to claim 18, into which further gene deficiencies have been crossed, for example for immuno-modulatory and immuno-regulatory gene functions, such as receptors or signal molecules.

20. Use of mice according to claims 18-19 as animal models for diseases connected with receptor EDG6.

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Abstract

The invention relates to G-protein coupled receptor EDG6 and its fragments, variants and mutations as well as its use. Fields of application of the invention are molecular biology, pharmacy and medicine.

The invention was based on the task of isolating and identifying a further member of the EDG receptor family and making it useful for a medicinal application.

The new human EDG6 receptor entails 384 amino-acids of Sequence 1 with seven transmembrane domains. The receptor possesses a possible N-terminal glycosylation point, three possible palmitoylation points positioned 12 to 15 amino-acids C-terminally from the seventh transmembrane domain as well as four possible C-terminal proteinkinase C-phosphorylation points.

The invention also relates to the use of the EDG6 receptors as well as its fragments, variants and mutations and, if applicable, its binding partners for therapeutic methods and treatments.

Norris, McLaughlin & Marcus, P.A.

220 East 42nd Street, 30th Floor
New York, NY 10017

If each inventor understands English, the Declaration and Power of Attorney below is suitable for use when filing a regular patent application and also when entering the national stage, in the case of an International application designating the USA under the PCT.

**COMBINED DECLARATION AND POWER OF ATTORNEY FOR
PATENT APPLICATION**

Attorney Docket No.
101195-45

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,
I believe I am the original, first and sole inventor (if only one name is listed below at 201) or an original,
first and joint inventor (if plural names are listed below at 201-205) of the subject matter which is claimed
and for which a patent is sought on the invention entitled

Human and Murine G-protein-coupled Edg6 Receptor (Endothelial Differentiation Gene) and Use of Same
the specification of which (check one)

☐ is attached hereto

☒ was filed on 10 September 1999

under Serial Number PCT/DE99/02871 and was amended on _____
(if applicable).

I hereby state that I have reviewed and understand the contents of the above-identified specification,
including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in
accordance with Title 37, Code of Federal Regulations, Section 1.56.

I list below any prior foreign application(s) for patent or inventor's certificate in respect of which foreign
priority benefits are claimed under 35 USC 119; and any prior foreign application(s) for patent or inventor's
certificate in respect of which such foreign priority rights are not claimed and which has a filing date before
that of any application in respect of which such foreign priority benefits are claimed:

| Application Number | Country | Filing Date (day, month, year) | Priority Claimed under 35 USC 119 |
|--------------------|---------|-----------------------------------|--|
| 198 43 240.2 | Germany | 11 September 1998 | YES: <input checked="" type="checkbox"/> NO: <input type="checkbox"/> |
| 198 46 979.9 | Germany | 13 October 1998 | YES: <input checked="" type="checkbox"/> NO: <input type="checkbox"/> |
| | | | YES: <input type="checkbox"/> NO: <input type="checkbox"/> |

I hereby claim the benefit under Title 35, United States Code, §119(e) of any United States provisional
application(s) listed below.

| Application No. | Filing Date |
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Mar-12-01 02:13pm From:Morris McLaughlin & Marcus

+212 808 0844

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| | | | |
|--|---------------------|--------------------------|------------------------|
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| Signature of Inventor 202 <i>Robert</i> | | <i>Robert</i> | Date 3/27/01 |
| Signature of Inventor 203 <i>Robert</i> | | <i>Robert</i> | Date 3/27/01 |
| Signature of Inventor 204 | | | Date |
| Signature of Inventor 205 | | | Date |

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101195-45

21. The following fees are submitted:

BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)) :

- ☐ Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO \$1,000.00
- ☒ International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO \$860.00
- ☐ International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO \$710.00
- ☐ International preliminary examination fee paid to USPTO (37 CFR 1.482) but all claims did not satisfy provisions of PCT Article 33(1)-(4) \$690.00
- ☐ International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(1)-(4) \$100.00

ENTER APPROPRIATE BASIC FEE AMOUNT =**CALCULATIONS PTO USE ONLY**

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Surcharge of \$130.00 for furnishing the oath or declaration later than ☐ 20 ☒ 30 months from the earliest claimed priority date (37 CFR 1.492 (e)).

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| CLAIMS | NUMBER FILED | NUMBER EXTRA | RATE |
|--------------------|--------------|--------------|-----------|
| Total claims | 20 - 20 = | 0 | x \$18.00 |
| Independent claims | 8 - 3 = | 5 | x \$80.00 |

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Multiple Dependent Claims (check if applicable). ☐

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TOTAL OF ABOVE CALCULATIONS =

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NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

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27387

PATENT TRADEMARK OFFICE

SIGNATURE

Bruce S. Londa

NAME

33-531

REGISTRATION NUMBER

March 9, 2001

DATE

Combined Declaration and Power of Attorney

101195-45

Page 2

I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith:

Bruce S. Londa (33,531) Lorimer P. Brooks (15,155) William R. Robinson (27,224)
Kurt G. Brisco (33,141) William C. Gerstenzang (27,552) Robert A. Hyde (46,354)
Davy E. Zoneraich (37,267) Mark A. Montana (44,948)

| | | | |
|--------------------|-----------------------|--------------------------|------------------------|
| 201 1-ED | Family Name | First Given Name | Second Given Name |
| | GRÄLER | Markus | |
| | City of Residence | State or Foreign Country | Country of Citizenship |
| | Berlin | Germany DEX | Germany |
| | Post Office Address | City | State & ZIP/Country |
| | Genthiner Strasse 30b | D-10785 Berlin | Germany |
| 202 2-ED | Family Name | First Given Name | Second Given Name |
| | BERNHARDT | Günter | |
| | City of Residence | State or Foreign Country | Country of Citizenship |
| | Berlin | Germany DEX | Germany |
| | Post Office Address | City | State & ZIP/Country |
| | Börnestrasse 38 | D-13086 Berlin | Germany |
| 203 3-ED | Family Name | First Given Name | Second Given Name |
| | LIPP | Martin | |
| | City of Residence | State or Foreign Country | Country of Citizenship |
| | Glienicke | Germany DEX | Germany |
| | Post Office Address | City | State & ZIP/Country |
| | Tulpenstrasse 31 | D-16548 Glienicke | Germany |
| 204 | Family Name | First Given Name | Second Given Name |
| | City of Residence | State or Foreign Country | Country of Citizenship |
| | Post Office Address | City | State & ZIP/Country |

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